Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and '*M. avium* subsp. *hominissuis*' for the human/porcine type of *M. avium*

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In an attempt to clarify the taxonomy of the *Mycobacterium avium* complex, the relationship between IS1245 RFLP, growth temperature, 16S rDNA signature sequences and the 16S–23S rDNA internally transcribed spacer (ITS) of 160 *M. avium*-complex isolates from different sources was investigated. All 70 isolates identified as *M. avium* by INNO-LiPA MYCOBACTERIA (Innogenetics, Belgium), a DNA probe test that targets the ITS, and by 16S rDNA analysis carried multiple copies of IS1245. Three isolates with multiple copies of IS1245 were identified by 16S rDNA analysis as *Mycobacterium intracellulare* and by LiPA as *M. intracellulare* ($n = 1$) and *M. avium*–*intracellulare* complex ($n = 2$). A dichotomy among the *M. avium* isolates was found on the basis of a C and a G signature nucleotide at position 228 of the 16S–23S rDNA spacer sequence, and this grouping was largely confirmed on the basis of similarities in IS1245 RFLPs. Strains with the characteristic three-band IS1245 'bird-type', as well as *M. avium* subsp. *silvaticum* or 'wood-pigeon' strains, invariably contained the C signature. A third characteristic that separated the *M. avium* bird-type isolates from *M. avium* isolates from humans and other mammals was growth-temperature tolerance: in contrast to bird isolates, human/porcine isolates grew at 24 and 45 °C. Based on differences in IS1245 RFLP, 16S–23S rDNA ITS and growth temperature, *M. avium* isolates originating from birds should be considered as a separate, evolutionarily conserved taxon. Because all *M. avium* isolates from birds are invariably of this type, the designation *M. avium* subsp. *avium* should be reserved for these bird-type strains. For clarity in the epidemiology of *M. avium*-related disease, isolates from humans and pigs with multibanded IS1245 RFLPs merit a separate designation. The designation '*M. avium* subsp. *hominissuis*' is suggested for this group of bacteria.

Keywords: *Mycobacterium avium* complex, IS1245 RFLP, 16S–23S rRNA spacer, LiPA

INTRODUCTION

Although bacteria of the *Mycobacterium avium* com-

Abbreviations: ITS, internally transcribed spacer; LiPA, line probe assay; MAC, *Mycobacterium avium* complex.

The GenBank accession numbers for the 16S–23S rDNA spacer nucleotide sequences determined in this work are AF315826 (Mac-J), AF315827 (Mac-K), AF315828 (Mac-L), AF315829 (Mac-M), AF315830 (Mac-N), AF315831 (Mac-O), AF315832 (Mac-P), AF315833 (Mac-Q), AF315834 (Mac-R), AF315835 (Mac-S), AF315836 (Mac-T), AF315837 (Mac-U), AF315838 (Mav-F) and AF315839 (Mav-G).

plex (MAC) cause a variety of diseases including tuberculosis-like disease in humans and birds, disseminated infections in AIDS patients and otherwise immunocompromised patients, lymphadenitis in humans and mammals and paratuberculosis (Johne's disease) in ruminants, they are ubiquitous in soil and water (Horsburgh, 1991; Inderlied *et al.*, 1993; Portaels, 1995; Von Reyn *et al.*, 1993). The MAC contains at least two clearly defined species: *M. avium* and *Mycobacterium intracellulare*. However, a significant proportion of the strains in this complex cannot

be classified in either species (Frothingham & Wilson, 1994; Wayne *et al.*, 1993).

Serotyping divides the MAC into 28 serovars. Reference strains with serovars 1–6, 8–11, 21 and 28 are considered to represent *M. avium*, while those with serovars 7, 12–20 and 25 are assigned to *M. intracellulare* (Saito *et al.*, 1990; Wayne *et al.*, 1993). However, up to a third of all isolates cannot be serotyped (Böddinghaus *et al.*, 1990). Based on biochemical and DNA analysis, *M. avium* can be further subdivided into subspecies corresponding to pathogenicity and host-range characteristics: *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis* (Thorel *et al.*, 1990). RFLP typing with the insertion sequences IS900 (Green *et al.*, 1989), IS901 (Kunze *et al.*, 1991) and IS1245 (Guerrero *et al.*, 1995) as probes can be used as markers for further subdivision among *M. avium* strains. IS900 is present specifically in *M. avium* subsp. *paratuberculosis*. The presence of IS901, as well as a specific three-banded IS1245 RFLP pattern, correlates well with the bird type of *M. avium* (Komijn *et al.*, 1999; Kunze *et al.*, 1991; Ritacco *et al.*, 1998; Van Soolingen *et al.*, 1998).

The 16S rDNA sequence is considered as the molecular 'gold standard' for mycobacterial species identification; a highly polymorphic region has been shown to contain species-specific DNA polymorphisms (Böddinghaus *et al.*, 1990; Kirschner *et al.*, 1993a). However, other semi-conserved genes, like *hsp65* (Devallois *et al.*, 1997; Leão *et al.*, 1999; Swanson *et al.*, 1997; Telenti *et al.*, 1993) and *rpoB* (Kim *et al.*, 2001), and the 16S–23S rDNA internally transcribed spacer (ITS) region (Abed *et al.*, 1995; Barry *et al.*, 1991; Glennon *et al.*, 1994; Portaels *et al.*, 1996; Roth *et al.*, 1998) have been shown to be more discriminating. The 16S–23S rDNA ITS sequence can be used to distinguish between the species *M. avium* and *M. intracellulare*. Furthermore, extended ITS sequence information has indicated that the MAC is genetically very heterogeneous. Also, among strains within the species *M. avium* and *M. intracellulare*, minor 16S–23S rDNA spacer sequence variation has been observed (De Smet *et al.*, 1995; Frothingham & Wilson, 1993, 1994; Roth *et al.*, 2000). The MAC can thus be subdivided into sequevars on the basis of differences in the 16S–23S rDNA ITS. However, the correlation of this observation with other genetic or phenotypic methods of grouping MAC isolates is unknown. To date, this technique has only been applied to reference strains and clinical isolates of human origin (De Smet *et al.*, 1995; Frothingham & Wilson, 1993, 1994).

Therefore, in this study, the concordance of several approaches to the grouping of MAC isolates originating from different sources was investigated. IS1245 RFLP typing, phenotypic characteristics, DNA sequence analysis of the 16S rDNA and the 16S–23S rDNA ITS region, AccuProbe (Gen-Probe) and the reverse hybridization line probe assay (LiPA) were

included to gain insights into the taxonomic grouping of MAC isolates from several hosts.

METHODS

Bacterial strains. A total of 160 mycobacterial isolates identified as belonging to the MAC on phenotypic grounds or by using the MAC AccuProbe (Gen-Probe) were investigated in this study. The sources and countries of origin of all isolates are summarized in Table 1. It should be noted that these isolates were selected on the basis of the sources of isolation, the presence/absence of IS1245 DNA and the IS1245-associated DNA polymorphism (Beggs *et al.*, 2000; Komijn *et al.*, 1999; Ritacco *et al.*, 1998; Schneider, 1998). Although MAC isolates with bird-type IS1245 RFLP patterns were almost invariably isolated from birds, we deliberately included bird-type isolates from other sources in this study (Komijn *et al.*, 1999; Ritacco *et al.*, 1998; Schneider, 1998). The MAC isolates from Belgium, India, Zaire (now the Democratic Republic of Congo), Italy, Australia, Benin and Finland were retrieved from the strain collection of the Mycobacteria Department of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium. The isolates from The Netherlands, Argentina and Zambia originated from the strain collection of the Mycobacteria Department at the National Institute of Public Health and the Environment (RIVM) in Bilthoven, The Netherlands. The cultures from the USA were kindly provided by Dr M. L. Beggs (University of Arkansas for Medical Sciences, Little Rock, AR, USA); Dr J. Falkinham (Virginia Polytechnic Institute and State University, VA, USA) provided the cigarette isolates. One of the most significant and comprehensive studies on the taxonomy of MAC bacteria was conducted by the International Working Group on Mycobacterial Taxonomy (IWGMT). Therefore, strains IWGMT 17 and IWGMT 26 were included in this study.

Phenotypic identification. Identification on the basis of biochemical tests and growth characteristics was performed as described previously (Komijn *et al.*, 1999; Thorel *et al.*, 1990).

Preparation of samples. Strains were kept at -70°C prior to testing. For identification procedures, cells were taken from the freezer and grown on Löwenstein–Jensen medium with (RIVM) or without (ITM) pyruvate. Cell suspensions were made in 100 μl Milli-Q water (Sigma-Aldrich) by taking a small loopful of bacteria either from the Löwenstein–Jensen slant or from an aliquot of the -70°C collection of bacterial isolates. The cells were inactivated at 95°C for 10 min and spun down at 10000 g for 1 min. Aliquots of the supernatant were used as targets for the tests.

DNA fingerprinting. DNA fingerprinting of MAC isolates was performed by RFLP typing using the insertion sequence IS1245 as a probe as described previously (Van Soolingen *et al.*, 1998). To facilitate computer-assisted analysis, internal and external molecular size markers and high-resolution gels (24 cm) were used as described in a proposal for standardization of IS1245 RFLP typing (Van Soolingen *et al.*, 1998). Computer-assisted analysis of the IS1245 fingerprints was carried out by using the BIONUMERICS software version 4.0 (Applied Maths). The RFLP patterns were compared by using the unweighted pair group method with arithmetic averages (UPGMA) clustering method and by using the Dice coefficient according to the instructions of the manufacturer of BIONUMERICS.

Table 1. Isolates and strains investigated

RIVM no.	IS1245 type	LiPA result	Origin	Country of origin
9800845	Bird type	<i>M. avium</i> C	Bird, wood pigeon	Belgium
9800846	Bird type	<i>M. avium</i> C	Bird, duck	Belgium
9800847	Bird type	<i>M. avium</i> C	Bird, goose	Belgium
9800850	Bird type	<i>M. avium</i> C	Bird, goose	Belgium
37229	Bird type	<i>M. avium</i> C	Bird, gull	Netherlands
160-74	Bird type	<i>M. avium</i> C	Bird, goose	Netherlands
75/219	Bird type	<i>M. avium</i> C	Bird, turkey	Netherlands
D61958	Bird type	<i>M. avium</i> C	Bird, fieldfare	Netherlands
D71076	Bird type	<i>M. avium</i> C	Bird, owl	Netherlands
16186	Bird type	<i>M. avium</i> C	Human	Netherlands
17689	Bird type	<i>M. avium</i> C	Human	Netherlands
9501279	Bird type	<i>M. avium</i> C	Mammal, pig	Argentina
9800849	Bird type	<i>M. avium</i> C	Mammal, cat	Belgium
9800854	Bird type	<i>M. avium</i> C	Mammal, cow	Belgium
9800855	Bird type	<i>M. avium</i> C	Mammal, cow	Belgium
V11	Bird type	<i>M. avium</i> C	Mammal, pig	Netherlands
IWGMT 17	Bird type	<i>M. avium</i> C	Reference	Netherlands
IWGMT 26	Bird type	<i>M. avium</i> C	Reference	Netherlands
9501268	Type 2	<i>M. avium</i> C	Human	Argentina
9601550	Type 2	<i>M. avium</i> C	Human	Netherlands
9601596	Type 2	<i>M. avium</i> C	Human	Netherlands
9602129	Type 2	<i>M. avium</i> C	Human	Netherlands
9701619	Type 3	<i>M. avium</i> T	Human	Netherlands
9600861	Type 4	<i>M. avium</i> C	Human	Netherlands
2000-00325	Type 5	<i>M. avium</i> C	Human	USA
2000-00337	Type 5	<i>M. avium</i> C	Human	USA
9601288	Type 6	<i>M. avium</i> C	Human	Netherlands
2000-00333	Type 6	<i>M. avium</i> T	Human	USA
9800858	Type 7	<i>M. avium</i> C	Mammal, pig	Belgium
9800851	Silvaticum type	<i>M. avium</i> C	Bird, penguin	Belgium
9801574	Silvaticum type	<i>M. avium</i> C	Bird, wood pigeon	Belgium
9801017	Cigarette type	<i>M. avium</i> C	Cigarettes	USA
9801024	Cigarette type	<i>M. avium</i> C	Cigarettes	USA
2000-00332	Positive	<i>M. avium</i> C	Human	USA
9800912	Positive	<i>M. avium</i> G	Environment, unknown	Finland
9800913	Positive	<i>M. avium</i> G	Environment, unknown	Finland
9800916	Positive	<i>M. avium</i> G	Environment, unknown	Finland
9800917	Positive	<i>M. avium</i> G	Environment, unknown	Finland
9800918	Positive	<i>M. avium</i> G	Environment, unknown	Finland
9600008	Positive	<i>M. avium</i> G	Human	Netherlands
9600670	Positive	<i>M. avium</i> G	Human	Netherlands
9601238	Positive	<i>M. avium</i> G	Human	Netherlands
9601246	Positive	<i>M. avium</i> G	Human	Netherlands
9601651	Positive	<i>M. avium</i> G	Human	Netherlands
9601815	Positive	<i>M. avium</i> G	Human	Netherlands
9601995	Positive	<i>M. avium</i> G	Human	Netherlands
9900525	Positive	<i>M. avium</i> G	Human	Netherlands
9900536	Positive	<i>M. avium</i> G	Human	Netherlands
9900537	Positive	<i>M. avium</i> G	Human	Netherlands
9900538	Positive	<i>M. avium</i> G	Human	Netherlands
2000-00326	Positive	<i>M. avium</i> G	Human	USA
2000-00327	Positive	<i>M. avium</i> G	Human	USA
2000-00328	Positive	<i>M. avium</i> G	Human	USA
2000-00329	Positive	<i>M. avium</i> G	Human	USA

Table 1 (cont.)

RIVM no.	IS/245 type	LiPA result	Origin	Country of origin
2000-00330	Positive	<i>M. avium</i> G	Human	USA
2000-00331	Positive	<i>M. avium</i> G	Human	USA
9800852	Positive	<i>M. avium</i> G	Mammal, cow	Belgium
9800853	Positive	<i>M. avium</i> G	Mammal, cow	Belgium
9800848	Positive	<i>M. avium</i> G	Mammal, dog	Belgium
9800856	Positive	<i>M. avium</i> G	Mammal, pig	Belgium
9800857	Positive	<i>M. avium</i> G	Mammal, pig	Belgium
9800859	Positive	<i>M. avium</i> G	Mammal, pig	Belgium
9800860	Positive	<i>M. avium</i> G	Mammal, pig	Belgium
9800861	Positive	<i>M. avium</i> G	Mammal, pig	Belgium
9800862	Positive	<i>M. avium</i> G	Mammal, pig	Belgium
9700615	Positive	<i>M. avium</i> G	Mammal, pig	Netherlands
9700622	Positive	<i>M. avium</i> G	Mammal, pig	Netherlands
9700657	Positive	<i>M. avium</i> G	Mammal, pig	Netherlands
9700662	Positive	<i>M. avium</i> G	Mammal, pig	Netherlands
9700672	Positive	<i>M. avium</i> G	Mammal, pig	Netherlands
2000-00334	Positive	<i>M. intracellulare</i>	Human	USA
2000-00335	Positive	MIC 2	Human	USA
2000-00336	Positive	MIC 2	Human	USA
9800903	Negative	<i>M. intracellulare</i>	Environment, soil	Italy
9600022	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600424	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600477	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600486	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600851	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600866	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600871	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600927	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600929	Negative	<i>M. intracellulare</i>	Human	Netherlands
9601067	Negative	<i>M. intracellulare</i>	Human	Netherlands
9601081	Negative	<i>M. intracellulare</i>	Human	Netherlands
9601103	Negative	<i>M. intracellulare</i>	Human	Netherlands
9601276	Negative	<i>M. intracellulare</i>	Human	Netherlands
9601510	Negative	<i>M. intracellulare</i>	Human	Netherlands
9601517	Negative	<i>M. intracellulare</i>	Human	Netherlands
9601795	Negative	<i>M. intracellulare</i>	Human	Netherlands
9701639	Negative	<i>M. intracellulare</i>	Human	Netherlands
9600592	Negative	<i>M. simiae</i>	Human	Netherlands
9601643	Negative	<i>Mycobacterium</i> sp.	Human	Netherlands
9700610	Negative	<i>M. xenopi</i>	Mammal, pig	Netherlands
9800920	Negative	MIC 1.1.b	Environment, soil	Benin
9601431	Negative	MIC 1.1.b	Human	Netherlands
9801875	Negative	MIC 1.1.b	Human	Netherlands
9701602	Negative	MIC 1.1.b	Human	Netherlands
9900293	Negative	MIC 1.1.b	Human	Zambia
9800911	Negative	MIC 1.2	Environment, dust	India
9800906	Negative	MIC 1.2	Environment, soil	Italy
9600270	Negative	MIC 1.2	Human	Netherlands
9600439	Negative	MIC 1.2	Human	Netherlands
9600515	Negative	MIC 1.2	Human	Netherlands
9601388	Negative	MIC 1.2	Human	Netherlands
9601800	Negative	MIC 1.2	Human	Netherlands
9801682	Negative	MIC 1.2	Human	Netherlands
9801789	Negative	MIC 1.2	Human	Netherlands

Table 1 (cont.)

RIVM no.	IS1245 type	LiPA result	Origin	Country of origin
9801833	Negative	MIC 1.2	Human	Netherlands
9900523	Negative	MIC 1.2	Human	Netherlands
9900810	Negative	MIC 1.2	Human	Netherlands
9800907	Negative	MIC 2	Environment, soil	Italy
9800908	Negative	MIC 2	Environment, soil	Italy
9600263	Negative	MIC 2	Human	Netherlands
9600468	Negative	MIC 2	Human	Netherlands
9600516	Negative	MIC 2	Human	Netherlands
9600548	Negative	MIC 2	Human	Netherlands
9600577	Negative	MIC 2	Human	Netherlands
9601127	Negative	MIC 2	Human	Netherlands
9601698	Negative	MIC 2	Human	Netherlands
9801790	Negative	MIC 2	Human	Netherlands
9602094	Negative	MIC 2	Human	Netherlands
9900022	Negative	MIC 2	Human	Netherlands
9900051	Negative	MIC 2	Human	Netherlands
9900146	Negative	MIC 2	Human	Netherlands
9900327	Negative	MIC 2	Human	Netherlands
9900344	Negative	MIC 2	Human	Netherlands
9900431	Negative	MIC 2	Human	Netherlands
9900438	Negative	MIC 2	Human	Netherlands
9900579	Negative	MIC 2	Human	Netherlands
9800897	Negative	MIC 3.1	Environment, unknown	Zaire
9600870	Negative	MIC 3.1	Human	Netherlands
9701604	Negative	MIC 3.1	Human	Netherlands
9701646	Negative	MIC 3.1	Human	Netherlands
9900005	Negative	MIC 3.1	Human	Netherlands
9800899	Negative	MIC 3.2	Environment, soil	Italy
9800902	Negative	MIC 3.2	Environment, soil	Italy
9601616	Negative	MIC 3.2	Human	Netherlands
9601940	Negative	MIC 3.2	Human	Netherlands
9800909	Negative	MIC 3.3	Environment, soil	Australia
9800898	Negative	MIC 3.3	Environment, plant	Zaire
9601404	Negative	MIC 3.3	Human	Netherlands
9601918	Negative	MIC 3.3	Human	Netherlands
9701605	Negative	MIC 3.3	Human	Netherlands
9900288	Negative	MIC 3.3	Human	Zambia
9800900	Negative	MIC 3.4	Environment, soil	Italy
9800905	Negative	MIC 3.4	Environment, soil	Italy
9801669	Negative	MIC 3.4	Human	Netherlands
9802080	Negative	MIC 3.4	Human	Netherlands
9600055	Negative	MIC 3.5	Human	Netherlands
9601002	Negative	MIC 3.5	Human	Netherlands
9800921	Negative	MIC 4	Environment, tap water	Belgium
9800914	Negative	MIC 4	Environment, unknown	Finland
9800915	Negative	MIC 4	Environment, unknown	Finland
9800919	Negative	MIC 4	Environment, unknown	Finland
9800910	Negative	MIC 4	Environment, water	India
9800901	Negative	MIC 4	Environment, soil	Italy
9800904	Negative	MIC 4	Environment, soil	Italy
9600915	Negative	MIC 4	Human	Netherlands
9801560	Negative	MIC 4	Human	Netherlands

16S rDNA analysis. Amplification of 700 bp of the 16S rDNA was performed using 15 pmol 16S-8F primer (5' AGAGT-TTGATCMTGGYTCAAG 3') and 10 pmol of the mycobacteria-specific primer 16S-M259 (5' TTTCACGAA-CAACGAGACAA 3') (Kirschner *et al.*, 1993b). The primers and 3 µl supernatant of the heat-inactivated suspension were added to a Ready-To-Go PCR bead (Amersham Pharmacia) and the reaction volume was adjusted to 25 µl using Milli-Q water (Sigma-Aldrich). The amplification mixtures were subjected to an initial denaturation of 3 min at 94 °C and 35 cycles of 94 °C for 20 s, 55 °C for 1 min and 72 °C for 1 min followed by a final extension period of 72 °C for 6 min. Amplified PCR products, expected to be of 700 bp, were analysed by 2% agarose gel electrophoresis and purified by using the PCR purification kit of Qiagen. The nucleotide sequence of this part of the 16S rDNA was determined on the forward strand by direct sequencing of the PCR product on an automated capillary DNA sequencer (model 310; Applied Biosystems), using fluorescence-labelled dideoxynucleotide terminators (ABI PRISM BigDye Terminator cycle sequencing ready reaction kit; Applied Biosystems). Analysis of the forward-strand sequence data was done using DNASTAR 5.0 software (DNASTAR). The species-specific (region A) and genus-specific (region B) portions of the 16S rDNA were analysed as described by Kirschner *et al.* (1993b). The 16S rDNA was compared with the known sequences described by Kirschner *et al.* (1993b) and with the BLAST database of the National Center for Biotechnology Information.

AccuProbe tests. AccuProbe tests (Gen-Probe) for the MAC and for *M. avium* and *M. intracellulare* separately were performed according to the instructions of the manufacturer.

Temperature growth range. A set of 30 MAC isolates selected on the basis of differences in IS1245 RFLP patterns, comprising seven bird-type strains, four multibanded strains, one type-2 strain, two cigarette isolates and 16 IS1245-negative isolates, were tested for their temperature ranges for growth. The isolates were grown at temperatures of 24, 30, 35, 37, 39, 42 and 45 °C on solid Löwenstein-Jensen medium supplemented with pyruvate. Growth was interpreted after 28 days.

LiPA. The LiPA is based on the reverse hybridization principle (Stuyver *et al.*, 1993). Specific oligonucleotides are immobilized as lines at known locations on a membrane strip and hybridized with a biotin-labelled PCR product. The hybrids formed are subsequently detected colorimetrically by adding a conjugate (alkaline phosphatase-labelled streptavidin) followed by a substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium). Probes were designed to hybridize in a hybridization solution of 3 × SSC and 0.2% SDS, at a hybridization temperature of 62 °C. In this study, three different strips were used, all based on the 16S–23S rDNA ITS as the target for amplification and detection.

First, strip 1 (INNO-LiPA MYCOBACTERIA; Innogenetics) was used to test all isolates. This test carries 14 probe lines. Twelve probe lines react specifically with *Mycobacterium tuberculosis* (MTB), *Mycobacterium kansasii* (MKA), *Mycobacterium xenopi* (MXE), *Mycobacterium goodii* (MGO), *M. avium* (MAV), *M. intracellulare* (MIN), *Mycobacterium scrofulaceum* (MSC) and the *Mycobacterium chelonae*–*Mycobacterium abscessus* complex (MCH). For both *M. kansasii* and the *M. chelonae*–*M. abscessus* complex, three probe lines each are included on the strips to distinguish

between environmental and possible clinically significant isolates. Two probe lines consist of general probes: one is termed 'MYC' for the genus *Mycobacterium* and one, termed 'MAIS', reacts with all MAC isolates, including *M. avium*, *M. intracellulare* and *M. scrofulaceum*. Furthermore, *Mycobacterium mageritense* and some *Mycobacterium haemophilum* isolates also react with this probe.

A second LiPA (strip 2) was used to differentiate isolates that reacted positively with the MAIS probe, but negatively with the specific *M. avium*, *M. intracellulare* or *M. scrofulaceum* probes on the INNO-LiPA MYCOBACTERIA strip. Eleven probe lines are present on strip 2: the *Mycobacterium* genus probe, specific probes for *M. mageritense* and *M. haemophilum* (since these particular species react with the MAIS probe on strip 1) and eight probes that generate specific probe patterns for different subgroups of MAC (see Fig. 2).

Finally, a third LiPA (strip 3), containing two probes and the *Mycobacterium* genus probe, was used to screen for the signature sequences at position 228 of the 16S–23S rDNA spacer region of *M. avium*. One probe was specific for C at this position; the other was specific for G (see Fig. 3).

Amplification was performed as follows: 1 µl of the supernatant of the heat-inactivated suspension was used as the target for the PCR, which was performed in a final volume of 50 µl containing 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 200 mM each dNTP and 1.5 U AmpliTaq polymerase (Perkin Elmer). The primers (P1 and P2), used at 50 pmol each per reaction, were biotinylated at their 5' ends. In the GeneAmp PCR System 9700 (Perkin Elmer), the following cycling protocol was applied: initial denaturation at 95 °C for 1 min and 40 cycles consisting of 30 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C, followed by immediate cooling to 4 °C. The mean length of the amplicon for MAC strains is 410 bp. This amplicon was used as the target for all three LiPA strips. For all three LiPAs, hybridization was performed according to the instructions of the INNO-LiPA MYCOBACTERIA kit.

ITS nucleotide sequencing. The nucleotide sequence of the 16S–23S rDNA ITS was determined on both strands by direct sequencing of the PCR product on an automated DNA sequencer (model 377; Applied Biosystems) using fluorescence-labelled dideoxynucleotide terminators (ABI PRISM BigDye Terminator cycle sequencing ready reaction kit; Applied Biosystems). The PCR was performed using primer P1, with the M13 (UP-21) universal primer site synthesized at its 5' end, and primer P2. Amplicons were purified by using the PCR purification kit of Qiagen. Sequencing reactions were performed with the universal M13 (UP-21) primer and P2. The new ITS sequences were aligned with those available in the databases (GenBank, National Center for Biotechnology Information) and further processed with GENEBASE version 1.0 (Applied Maths). Ambiguities in published databases were considered as identities.

RESULTS

IS1245 RFLP typing

All 70 mycobacterial isolates from six different sources (Table 2) identified as *M. avium* on the basis of 16S rDNA sequence analysis and the INNO-LiPA MYCOBACTERIA test contained IS1245 DNA as shown in

Table 2. Source, specification and geographical origin of selected isolates based on IS1245 RFLP type, LiPA strip results and temperature range for growth

LiPA strip test results are indicated as follows. Strip 1: 1, MAIS; 2, *M. avium*; 3, *M. intracellulare*; 4, *M. xenopi*; 5, *Mycobacterium* sp. Strip 2: 1, MIC 1.1.b; 2, MIC 1.2; 3, MIC 2; 4, MIC 3.1; 5, MIC 3.2; 6, MIC 3.3; 7, MIC 3.4; 8, MIC 3.5; 9, MIC 4, Strip 3: 1, *M. avium* C; 2, *M. avium* G; 3, *M. avium* T. The number of isolates/strains tested for their growth temperature range is indicated.

Specification*	IS/245 type	LiPA strip 1					LiPA strip 2									LiPA strip 3			Growth temperature (°C)					
		1	2	3	4	5	1	2	3	4	5	6	7	8	9	1	2	3	n	24	30–37	39	42	45
Mammal																								
1 cat ¹ , 2 cow ¹ , 2 pig ^{2,3}	Bird type		5												5			2	—	+	+	+	—	
1 pig ¹	Type 7		1												1									
2 cow ¹ , 11 pig ^{6×1.5×2} , 1 dog ¹	Multibanded		14														14							
1 pig ²	Negative				1																			
Bird																								
1 duck ¹ , 3 goose ^{2×1.2} , 1 fieldfare ² , 1 gull ¹ , 1 owl ¹ , 1 turkey ¹ , 1 wood pigeon ²	Bird type		9												9			3	—	+	+	+	—	
1 penguin ¹ , 1 wood pigeon ¹	Silvaticum type		2												2									
Cigarettes																								
2 tobacco ¹²	Cigarette type		2												2			2	+	+	+	+	+	
Environment																								
5 unknown ⁹	Multibanded		5														5	3	+	+	+	+	+	
1 dust ⁴ , 1 plant ⁵ , 12 soil ^{10×6,1×7,1×8} , 2 tap water ^{1,4} , 4 unknown ^{3×9,1×5}	Negative	19		1			1	2	2	1	2	2	2	7										
Human																								
2 patients ²	Bird type		2												2			2	—	+	+	+	—	
4 patients ²	Type 2		4												4			1	+	+	+	+	+	
1 patient ²	Type 3		1														1							
1 patient ²	Type 4		1												1									
2 patients ¹⁰	Type 5		2												2									
2 patients ^{2,10}	Type 6		2												1		1							
21 patients ^{3,11×2,10×10}	Multibanded	2	18	1					2						1	17		1	+	+	+	+	+	
17 patients ²	Negative			17														5	+	+	+	+	—	
49 patients ²	Negative	47				2	4	10	17	4	2	4	2	2				11	+	+	+ / —	—	—	
Reference																								
IWGMT 17, IWGMT 26	Bird type		2												2									
Totals		68	70	19	1	2	5	12	21	5	4	6	4	2	9	32	36	2	30					

*Numbers and sources of isolates are indicated. Country of origin is indicated by superscript numbers as: 1, Belgium; 2, The Netherlands; 3, Argentina; 4, India; 5, Zaire; 6, Italy; 7, Australia; 8, Benin; 9, Finland; 10, USA (Little Rock, AR); 11, Zambia; 12, USA (Virginia).

RFLP typing (Fig. 1). In addition, one of 19 isolates identified as *M. intracellulare* on LiPA and two of 68 isolates identified as MAIS on LiPA exhibited multibanded patterns in IS1245-based RFLP typing.

The IS1245 bird-type patterns, showing three bands, of 8.7, 3.2 and 2.9 kb (Bono *et al.*, 1995; Ritacco *et al.*, 1998), were found in two *M. avium* isolates from cows, two from pigs, one from a cat, nine from different bird species, two from humans and in two reference isolates. The two *M. avium* subsp. *silvaticum* strains, isolated from a penguin and a wood pigeon, showed only a one-band difference with respect to the IS1245 bird-type pattern, as described previously (Bono *et al.*, 1995). The two *M. avium* isolates from cigarettes contained two and four copies of IS1245, and the respective IS1245 RFLP patterns were not found among strains from other sources. However, the few bands of the cigarette isolates matched with bands of one particular group (type 2) of *M. avium* isolates from humans. Ten *M. avium* strains from humans and one from a pig contained other IS1245 RFLP patterns,

named type 2 to type 7. Multibanded and highly polymorphic patterns were encountered for 14 mammal isolates, five environmental isolates and 18 clinical isolates, but not in bird isolates (Fig. 1, Table 2).

The IS1245 RFLP patterns were assembled in two large groupings with only 20% mutual similarity. All isolates in the first group exhibited multibanded (13–39 copies) IS1245 RFLP patterns. In the second group, all isolates except one exhibited one to nine copies of IS1245. The exceptional strain contained 13 copies of the IS1245 element (type 4) (Fig. 1).

INNO-LiPA MYCOBACTERIA (LiPA strip 1)

For all 160 isolates that were identified as MAC on phenotypic grounds or with the MAC AccuProbe, the spacer region was amplified and analysed by reverse hybridization using the commercial INNO-LiPA MYCOBACTERIA strip. All reacted with the general *Mycobacterium* probe on the strip. Furthermore, all except

IS1245 RFLP	No.	RFLP type	LiPA
	<i>n</i> = 1	Multibanded	<i>M. intracellulare</i>
	<i>n</i> = 2	Multibanded	MAIS
	<i>n</i> = 36	Multibanded	<i>M. avium</i> (G)
	<i>n</i> = 1	Multibanded	<i>M. avium</i> (C)
	<i>n</i> = 1	Type 4	<i>M. avium</i> (C)
	<i>n</i> = 4	Type 2	<i>M. avium</i> (C)
	<i>n</i> = 1	Type 7	<i>M. avium</i> (C)
	<i>n</i> = 18	Bird type	<i>M. avium</i> (C)
	<i>n</i> = 2	Silvaticum type	<i>M. avium</i> (C)
	<i>n</i> = 1	Type 3	<i>M. avium</i> (T)
	<i>n</i> = 2	Cigarette type	<i>M. avium</i> (C)
	<i>n</i> = 2	Type 5	<i>M. avium</i> (C)
	<i>n</i> = 1	Type 6	<i>M. avium</i> (T)
	<i>n</i> = 1	Type 6	<i>M. avium</i> (C)

Fig. 1. Representative IS1245 RFLP patterns of the MAC isolates included in this study and their LiPA results.

Fig. 2. Representative LiPA patterns on strip 2. Strip 2 allows further differentiation of isolates with an MAIS pattern in the INNO-LiPA MYCOBACTERIA test (strip 1 illustrated). Different subgroups can be distinguished: MIC 1.1.b (1), MIC 1.2 (2), MIC 2 (3), MIC 3.1 (4), MIC 3.2 (5), MIC 3.3 (6), MIC 3.4 (7), MIC 3.5 (8) and MIC 4 (9); in addition, two species-specific probes are present, one for *M. malmoense* (10) (probe MML) and one for *M. haemophilum* (11) (probe MHP).

three reacted with the MAIS probe: two isolates reacted only with the general *Mycobacterium* probe and were therefore classified as unidentified mycobacteria, whereas one isolate reacted with the *M. xenopi* probe on the strip, although the strain had previously been identified phenotypically as MAC (Komijn *et al.*, 1999). Seventy isolates, including two isolates belonging to *M. avium* subsp. *silvaticum*, were identified as *M. avium* by LiPA and invariably contained IS1245 DNA. Of the remaining 87 isolates, 19 were identified by LiPA as *M. intracellulare* and 68 reacted exclusively with the MAIS probe. All these 87 isolates that did not react with the *M. avium* probe were negative in the IS1245 RFLP hybridization, except for three: one was identified by both the AccuProbe and LiPA test as *M. intracellulare*, and two were *M. intracellulare* AccuProbe-positive and identified by LiPA as MAIS (Table 2).

LiPA strip 2

The 68 MAC isolates that reacted only with the MAIS probe on the INNO-LiPA MYCOBACTERIA strip were further analysed on strip 2. This research strip was designed to allow further differentiation of MAC isolates (W. Mijs and R. Rossau, unpublished). According to the probe patterns obtained, these 49 clinical and 19 environmental isolates could be subdivided into nine different groups in the MAC (Fig. 2, Tables 1–4).

Fig. 3. Representative LiPA patterns on strip 3. Lines C and G detect a single mismatch, C or G, respectively, thereby screening for the signature sequence at position 228 in the *M. avium* spacer: *M. avium* 'bird' – C (1), *M. avium* 'pig' – G (2), *M. avium* – T (3) are shown.

LiPA strip 3

A third LiPA research strip (Fig. 3) was used to enable differentiation of the 70 *M. avium* isolates based on the known polymorphism at position 228 in the spacer

Table 3. Nucleotide mismatches in the ITS regions of all described and new sequevars of *M. avium*, *M. intracellulare* and the MAC

Nucleotides are indicated relative to the *M. intracellulare* ITS reference sequence (Min-A, accession no. L07859). Only those positions at which nucleotide changes were observed are listed. Identities are indicated by dots (.); gaps are indicated by dashes (-).

Sequevar*	LiPA result†	n‡	21	22	23	27	33	34	38	39	53	54	55	69	71	72	73	74	76	83	84	94	96	99	137	136	139	140	149	159	217	218	221	222	223	224	226	230	231	232	234	236	238	239	241	245	246	248	249	264	266	270	271	272	273	275		
Min-A	(<i>M. intracellulare</i>)		C	T	C	T	G	G	C	G	T	C	C	C	G	G	G	G	C	A	C	–	G	T	C	G	A	T	A	T	G	C	A	T	A	G	C	A	G	–	G	T	A	T	C	C	G	C	G	T	C	–	G	G	T	T		
Min-A	<i>M. intracellulare</i>	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Min-D	(<i>M. intracellulare</i>)		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Min-C	(MIC 1.1.b)		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Min-C	MIC 1.1.b	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Min-B	(MIC 1.1.b)		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Mac-B	(MIC 1.2)		.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-B	MIC 1.2	1	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-C	(MIC 1.2)		.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-D	(MIC 1.2)		.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-D	MIC 1.2	1	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-J	MIC 1.2	1	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-K	MIC 4	1	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Mac-L	MIC 1.2	1	T	C	.	.	.	.	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-M	MIC 1.2	1	T	C	.	.	.	.	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-I	(MIC 4)		T	C	.	.	.	.	.	.	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-N	MIC 1.2	1	.	.	.	.	.	.	.	T	.	A	A	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-O	MIC 4	1	.	.	.	.	.	.	.	C	.	T	.	A	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-A	(MIC 2)		.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-A	MIC 2	5	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	A	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-P	MIC 2	1	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-E	(MIC 3.4)		.	.	.	.	.	A	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-E	MIC 3.4	2	.	.	.	.	.	A	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-F	(MIC 3.4)		.	.	.	C	.	A	.	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			
Mac-H	(MIC 3.4)		.	.	.	.	.	A	.	A	T	.	A	A	A	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-Q	MIC 3.3	2	.	.	T	.	.	A	.	.	T	.	A	A	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-R	MIC 3.3	1	.	.	T	.	.	A	A	.	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-S	MIC 3.5	2	T	.	.	.	.	A	T	.	.	T	.	A	A	A	A	.	.	.	.	.	–	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-T	MIC 3.2	2	T	C	.	.	.	.	.	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mac-U	MIC 3.1	1	.	.	T	.	.	A	A	.	.	T	.	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-A	(<i>M. avium</i> C)		.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-A	<i>M. avium</i> C	20	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-G	<i>M. avium</i> C	1	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-C	(<i>M. avium</i> C)		.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-D	(<i>M. avium</i> C)		.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-B	(<i>M. avium</i> G)		.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-B	<i>M. avium</i> G	6	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-E	(<i>M. avium</i> T)		.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Mav-F	<i>M. avium</i> T	2	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		

* New sequevars are indicated in bold.

† Theoretically derived LiPA patterns are indicated in parentheses.

‡ Number of isolates tested in LiPA.

Table 4. Relationship between *M. intracellulare* AccuProbe test results, 16S rDNA sequencing and analysis of non-*M. avium* MAC isolates

For 16S rDNA region A, types represent: A, *M. intracellulare*; B, *M. intracellulare* serovar 18; C, *M. intracellulare* serovar 7 (according to Kirschner *et al.*, 1993b); D, IWGMT 90242 (according to Wayne *et al.*, 1996); E, identical to type C except for an extra T at position 219 (type E has not been described previously). For 16S rDNA region B, types represent: A, *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. intracellulare*, *Mycobacterium szulgai*, *M. malmoeense* and *Mycobacterium asiaticum*; B, *Mycobacterium marinum*, *M. haemophilum*, *Mycobacterium ulcerans* and *M. intracellulare* (according to Kirschner *et al.*, 1993b); C, IWGMT 90233 according to Wayne *et al.* (1996).

<i>M. intracellulare</i> AccuProbe test result	16S rDNA		LiPA test result									Total
	Region A	Region B	<i>M. intracellulare</i>	MIC 1.1.b	MIC 1.2	MIC 2	MIC 3.1	MIC 3.2	MIC 3.3	MIC 3.4	MIC 4	
Positive	A	A	11	2								13
	A	B				18						18
	B	A		1								1
	D	A		1								1
	B	B				1						1
Negative	B	A					2	2	1		1	6
	B	B			6		1					7
	B	C			2							2
	C	B							2			2
	D	A			1					2		3
	E	A									1	1
Totals			11	4	9	19	3	2	3	2	2	55

sequence (either C or G), previously described respectively as sequevars Mav-A and Mav-B (Frothingham & Wilson, 1993). The distinction into two groups on the basis of the similarities in IS1245 RFLP patterns was almost invariably confirmed by the presence of either C or G at position 228 in the spacer sequence. Thirty-six *M. avium* isolates with multibanded and polymorphic IS1245 patterns reacted with the G probe, whereas 31 *M. avium* isolates of the second group reacted with the C probe, indicating the presence of G or C, respectively, at position 228 in the spacer sequence (Table 2). One exceptional *M. avium* isolate with a multibanded IS1245 RFLP pattern reacted repeatedly with the C probe. Two patient isolates, respectively with one and two copies of IS1245, did not react with the G or C probe.

16S–23S rDNA spacer sequences

To validate the LiPA results further, the ITS regions from 64 of the 160 *Mycobacterium* isolates were sequenced. For one of the two isolates that reacted only with the general *Mycobacterium* probe, a mycobacterial spacer was found that did not match any described or published sequence. For the other isolate, a *Mycobacterium simiae* spacer sequence was found. Five *M. intracellulare* isolates showed identical spacer sequences, previously named Min-A (Frothingham & Wilson, 1993). Of the 29 *M. avium* isolates sequenced, 20 had C at position 228 (Mav-A) and six had G at this position (Mav-B), in accordance with the LiPA research strip 3 analysis. Two isolates that did not react with the C and G probe on the LiPA research strip 3 had T at this position as well as T₇, rather than T₆ at positions 260–266. The latter signature sequence was described earlier for sequevar Mav-E (De Smet *et al.*, 1995). However, our two *M. avium* T isolates differed from the Mav-E sequevar at position 229, and were

therefore termed Mav-F. Furthermore, one of the *M. avium* C isolates differed from the Mav-A sequevar by having T₅ at positions 260–266; consequently, it was named sequevar Mav-G. These two new *M. avium* sequevars (Mav-F, Mav-G) were deposited in the GenBank database under accession numbers AF315838 and AF315839. Of the remaining 28 MAC isolates that reacted only with the MAIS probe on strip 1, 13 matched a sequence described in the literature [Min-C (*n* = 4), Mac-A (*n* = 5), Mac-B (*n* = 1), Mac-D (*n* = 1), Mac-E (*n* = 2)] and 15 isolates could be divided into 12 different new sequevars (Mac-J to Mac-U, AF315826–AF315837). Observed mismatches between the various MAC spacer sequences and the *M. intracellulare* reference sequence (Min-A; GenBank accession no. L07859) are summarized in Table 3.

Detailed analysis of non-*M. avium* MAC isolates

Fifty-five of the 68 MAIS isolates identified by means of the INNO-LiPA MYCOBACTERIA strips (Table 2) were subjected to the *M. intracellulare* AccuProbe test and 16S rDNA sequence analysis (Table 4). The *M. intracellulare* AccuProbe-positive isolates were identified by LiPA as *M. intracellulare* (*n* = 11), MIC 1.1.b (*n* = 4) or MIC 2 (*n* = 19). All isolates having other MIC ITS types (*n* = 21) did not react with the *M. intracellulare* AccuProbe test. For clarity, the different DNA sequences in the 16S rDNA species- and genus-specific regions are indicated by letter combinations (explained in the legend to Table 4). All 11 isolates identified by INNO-LiPA as *M. intracellulare*, as well as two MIC 1.1.b isolates, revealed the combination A–A. The combination A–B was found exclusively in the 19 isolates that were identified by LiPA as MIC 2, with one exception. This exceptional strain showed the newly described ITS sequevar Mac-P. None of the *M.*

intracellulare AccuProbe-negative isolates had a species-specific region of type A.

Temperature growth range

A selection of 30 MAC isolates was tested for their temperature growth range at seven temperatures ranging from 24 to 45 °C. The bird-type isolates did not grow at temperatures of 24 or 45 °C. The multicopy strains, type 2, and the cigarette-type strains were able to grow over the entire temperature range of 24–45 °C. The IS1245-negative isolates could be divided into two groups based on temperature growth range: one was unable to grow at 45 °C and another was unable to grow at 42 or 45 °C. These groups were identified by INNO-LiPA MYCOBACTERIA as *M. intracellulare* and MAIS, respectively.

DISCUSSION

Previous attempts to classify organisms of the MAC have only highlighted the vast heterogeneity within this group. In the present study, we combined several previously described approaches and compared these with new molecular assays such as the reverse line-blot hybridization used in the LiPA system.

Previous studies showed that the IS1245 insertion element is specific for *M. avium*. In this study, all 70 *M. avium* isolates identified by phenotypic testing and analysis of the 16S rDNA and the ITS carried the IS1245 insertion element. All of the *M. avium* isolates reported by Beggs *et al.* (2000) as lacking IS1245 DNA that were retested in our laboratory appeared to hybridize with the IS1245 probe (P. de Haas, D. van Soolingen and W. Mijls, unpublished data). However, three isolates that were negative with the *M. avium* probe on the INNO-LiPA MYCOBACTERIA strip harboured multiple copies of IS1245, and were respectively identified as *M. intracellulare* and MAIS by LiPA and as *M. intracellulare* by the AccuProbe test.

Both the phenotypic and the genetic information presented here reveals that *M. avium* isolates from birds constitute a separate taxon within the MAC. In an earlier study, we found that both the IS901 and IS1245 RFLPs of bird isolates are highly conserved (Ritacco *et al.*, 1998): all 29 *M. avium* isolates from 23 different bird species invariably belonged to the bird-type RFLP grouping within the complex, while this type of strain was hardly found in other reservoirs. In a population-based study in The Netherlands in 1997, none of the 195 *M. avium* isolates examined from humans was of the bird type (Schneider, 1998).

In this study, additional reasons are provided to support a separate designation for *M. avium* isolates from birds. Firstly, isolates originating from pigs and humans still grow at 45 °C while bird types fail to grow at this temperature. Secondly, a signature nucleotide C was found at position 228 of the 16S–23S rDNA ITS region for all *M. avium* isolates that had a characteristic three-banded IS1245 RFLP pattern. *M. avium* subsp.

silvaticum, the ‘wood-pigeon’ variant of *M. avium*, is closely related to the ‘bird-type’ strains on the basis of IS1245 RFLP, and also contains the same C signature nucleotide. In contrast, a G signature nucleotide was found for isolates that harboured highly polymorphic and multibanded IS1245 RFLP patterns, with only one exception, an isolate that had a multibanded IS1245 RFLP pattern while harbouring a C signature sequence.

Frothingham & Wilson (1994, 1993) as well as De Smet *et al.* (1995) respectively found previously that 3/11 and 2/25 human clinical isolates harboured a signature nucleotide C (which they termed sequevar Mav-A) and that respectively 8/11 and 22/25 human isolates had G (which they termed sequevar Mav-B). Also, as described by De Smet *et al.* (1995), 1/25 human isolates had T at this signature position, as well as T₇ rather than T₆ at positions 260–266, which was termed Mav-E. In our study, 11/30 human clinical isolates harboured a signature nucleotide C, 17/30 had G and 2/30 had T.

The development of a LiPA (strip 3) based on the position 228 signature nucleotide indicates that it is possible to screen for different subtypes of *M. avium* with this methodology. Indeed, the actual source of *M. avium* infections in humans and pigs remains unclear. The differences between isolates from birds and humans presented here and previously (Komijn *et al.*, 1999; Ritacco *et al.*, 1998) should eliminate the misconceptions about birds being the source of *M. avium* infections in humans. In the epidemiology of *M. avium*-related infection, there is considerable confusion regarding the origin of such infections. To clarify the epidemiology of *M. avium*-related infections, we therefore propose to reserve the designation *M. avium* subsp. *avium* for the genetically highly conserved, bird-type isolates. These isolates are characterized by a specific IS1245 RFLP consisting of three bands, of 8·7, 3·2 and 2·9 kb. The 16S–23S rDNA spacer region contains the specific C signature sequence, and these bird types do not grow at temperatures of 24 or 45 °C. Furthermore, *M. avium* subsp. *avium* isolates exhibit a highly conserved IS901 RFLP containing 7–11 copies (Ritacco *et al.*, 1998) and they invariably belong to serovars 1, 2 and 3 (Ritacco *et al.*, 1998). *M. avium* subsp. *silvaticum* is highly related to *M. avium* subsp. *avium* and has been isolated mainly from wood pigeons. These strains exhibit a shift of one band in the IS1245 RFLP pattern, of 8·7 to 2·2 kb, in comparison with the *M. avium* subsp. *avium* IS1245 RFLP pattern. Furthermore, *M. avium* subsp. *silvaticum* strains have a conserved IS901 RFLP pattern similar to that of *M. avium* subsp. *avium*. In contrast to *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum* strains are mycobactin-dependent (Bono *et al.*, 1995).

If another designation were to be chosen for the *M. avium* strains with multibanded IS1245 RFLP patterns found in humans and pigs (Kirschner *et al.*, 1993a), it would become clear to clinicians that *M. avium*

infections in humans in most cases do not originate from birds. Because the source of *M. avium* infections in humans and pigs is unclear at the moment, we propose the designation '*M. avium* subsp. *hominissuis*' for this group of *M. avium* bacteria in the complex. This designation should be used for *M. avium* isolates that do not exhibit the specific characteristics of the bird-type isolates. '*M. avium* subsp. *hominissuis*' isolates are genetically much more divergent than *M. avium* subsp. *avium* isolates: most (but not all) *M. avium* isolates from humans and pigs contain multiple copies of IS1245 (Ritacco *et al.*, 1998) and the associated RFLP is highly polymorphic, the 16S–23S rDNA spacer region contains the specific G signature sequence and these pig/human isolates grow over the complete temperature range of 24–45 °C. Furthermore, IS901 has been found only incidentally in '*M. avium* subsp. *hominissuis*' isolates and was, in that case, not associated with the characteristic IS901 RFLP found in *M. avium* subsp. *avium* (Ritacco *et al.*, 1998).

The IS1245-negative isolates were further analysed with LiPA, with AccuProbe and by sequencing of 16S rDNA signature sequences and the ITS. Although the isolates were identified as being MAC, based on morphology and biochemical testing, two isolates turned out to be '*Mycobacterium* sp.' and one was identified as *M. xenopi* by the INNO-LiPA MYCOBACTERIA test. For the *Mycobacterium* sp. isolates, ITS sequencing revealed an *M. simiae* spacer and an 'undefined' *Mycobacterium* spacer, proving that these isolates are not MAC. Similar anomalies have been noted before (De Smet *et al.*, 1995, 1996), highlighting the limitations of biochemical testing and showing the advantage of DNA-based tests such as LiPA for identification.

All 'true' MAC isolates, including *M. avium* and *M. intracellulare*, reacted with the MAIS probe in the LiPA test. However, two isolates that harboured identical spacer sequences (designated MIC.3.5) gave a very weak reaction with this probe. Sequencing of the spacer region showed them to be identical, showing at least six mismatches with any other spacer sequence. The presence of nucleotide mismatches in the probe region of the MAIS probe explained the weak reaction. Among the MAIS-positives, 19 isolates hybridized with the *M. intracellulare*-specific MIN probe on the INNO-LiPA MYCOBACTERIA strip. Sequencing of the spacers of five of them confirmed that they harboured 100%-identical spacer sequences, identical to the described sequevar Min-A. This sequevar is considered as 'true' *M. intracellulare* by the INNO-LiPA MYCOBACTERIA test.

The LiPA 'MAIS' isolates (MAIS probe-positive; *M. avium*- and *M. intracellulare*-negative) were further tested with a second strip (strip 2) and divided into 'MIC' groups – referring to '*M. intracellulare* complex', since MAC strains seem to be more related to *M. intracellulare* (99.3–91.1%) than to *M. avium* (94.3–89.4%) at the level of the ITS. According to previous

studies, three main groups with eight subgroups (named MIC 1.1.b, 1.2, 2, 3.1, 3.2, 3.3, 3.4 and 4) could be defined on the basis of probe patterns (data not shown). Some of them were equivalent to earlier described sequevars. In this study, these probe patterns and one new probe pattern were found (MIC 3.5).

Sequencing of the spacer region shows that *M. avium* forms a clearly defined species within the MAC group. The remaining MAC isolates, including *M. intracellulare*, form a very heterogeneous group, with at least 30 different sequences currently known to differ from one another by between one and more than 20 nucleotides (this study; W. Mijs, F. Portaels and R. Rossau, unpublished; De Smet *et al.*, 1995; Frothingham & Wilson, 1993, 1994). Sequencing confirmed all LiPA strip-2 patterns, but the groupings derived from probe patterns did not always correspond with those derived from spacer sequences. For example, one MIC 4 isolate showed 99.6% similarity to MIC 1.2 isolates, because the one nucleotide mismatch was situated in one probe region. This illustrates that, although the probe patterns can be considered as a type of fingerprint, the LiPA MIC groups do not match their phylogenetic relationship exactly. Fine-tuning of these probes and relating them to clinical relevance may improve these inaccuracies.

Comparison of two signature-sequence regions in the 16SrDNA, the AccuProbe results, INNO-LiPA MYCOBACTERIA and the MIC groupings found with the second LiPA strip revealed the following correlations: (i) all isolates positive with the *M. intracellulare* probe on LiPA and all isolates having a MIC 1.1.b or MIC 2 LiPA pattern were positive for the *M. intracellulare* AccuProbe; (ii) all isolates having other MIC patterns reacted only with the AccuProbe for the MAC. These findings may indicate that the INNO-LiPA MYCOBACTERIA strip identifies a cluster too narrow to include all *M. intracellulare* strains. Indeed, sequencing of the spacer region of MIC 1.1.b isolates revealed 99.3% similarity to the reference spacer of *M. intracellulare*, and was previously described as sequevar Min-C. However, sequencing of the spacer region of MIC 2 isolates revealed only 93.2% similarity to the reference spacer. The latter finding may indicate that the cluster that is now considered as *M. intracellulare* on the basis of AccuProbe tests is too wide. When comparing these results with those of the 16S rDNA signature sequencing, it should be noted that, for *M. intracellulare* isolates, neither the AccuProbe results nor the LiPA results correlate fully with those obtained by 16S polymorphic region sequencing. Isolates with the same 16S sequence may give different AccuProbe reactivities and may differ on the basis of the ITS sequence and vice versa (Table 3).

Conclusion

The aim of this study was to clarify the taxonomy of the MAC. We found that IS1245 fingerprinting and the use of the INNO-LiPA MYCOBACTERIA strip based

on the 16S–23S rDNA ITS are useful tools for clarifying the taxonomy of the MAC. Using these techniques, *M. avium* isolates can be distinguished easily from other MAC isolates by the presence of the IS1245 insertion element, as well as by a positive reaction with the *M. avium* probe on the INNO-LiPA MYCOBACTERIA strip, a commercially available reverse hybridization assay.

Fingerprinting by IS1245 RFLP and sequencing of the 16S–23S rDNA spacer region also revealed a difference between *M. avium* strains isolated from birds and those isolated from pigs. A specific RFLP pattern and spacer sequence were found for bird types, while other patterns or sequences were found for pig types. The use of two spacer probes easily allows screening for this signature sequence. This may help to pinpoint the reservoir of environmental *M. avium* strains that tend to cause infections in both humans and pigs. The genotypic differences between bird isolates and pig/human isolates were confirmed by phenotypic differences such as growth temperature, leading us to suggest the designation of subspecies *M. avium* subsp. *avium* and '*M. avium* subsp. *hominissuis*', respectively.

Although *M. avium* can be distinguished easily from other MAC isolates, and even be subdivided into different (sub)species, it is still difficult to apply the name *M. intracellulare* to a specific species, since techniques based on different molecular markers generate different results. Further study is therefore required.

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